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The conserved role and divergent regulation of *foxa*, a pan-eumetazoan developmental regulatory gene

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ABSTRACT

Foxa is a forkhead transcription factor that is expressed in the endoderm lineage across metazoans. Orthologs of *foxa* are expressed in cells that intercalate, polarize, and form tight junctions in the digestive tracts of the mouse, the sea urchin, and the nematode and in the chordate notochord. The loss of *foxa* expression eliminates these morphogenetic processes. The remarkable similarity in *foxa* phenotypes in these diverse organisms raises the following questions: why is the developmental role of Foxa so highly conserved? Is *foxa* transcriptional regulation as conserved as its developmental role? Comparison of the regulation of *foxa* orthologs in sea urchin and in *Caenorhabditis elegans* shows that *foxa* transcriptional regulation has diverged significantly between these two organisms, particularly in the cells that contribute to the *C. elegans* pharynx formation. We suggest that the similarity of *foxa* phenotype is due to its role in an ancestral gene regulatory network that controlled intercalation followed by mesenchymal-to-epithelial transition. *foxa* transcriptional regulation had evolved to support the developmental program in each species so *foxa* would play its role controlling morphogenesis at the necessary embryonic address.

Introduction

Regulatory genes play a major role in the evolution of diverse body plans as they control the differential expression of regulatory and structural genes (Davidson, 2006; Ben-Tabou de-Leon and Davidson, 2007; Ben-Tabou de-Leon and Davidson, 2009). There are examples for evolutionary rearrangement of the architecture of developmental gene regulatory networks (GRNs) that result in change in the organism morphological features (Hinman and Davidson, 2007; Gao and Davidson, 2008; Fraser et al., 2009; Lemons et al., 2010) and examples for conservation of morphological features that relate to preservation of ancestral developmental GRN (see *e.g.*, (Hinman et al., 2003; Fraser et al., 2009; McCauley et al., 2010)).

A common feature of developmental GRNs is that the specific combinations of transcription factors define the specification state of a cell, and not individual transcription factors (Davidson, 2006). Yet, there are certain regulatory genes that seem to be involved in a similar developmental task in a wide range of organisms. Examples for such regulatory genes are the transcription factor Pax6 that is involved in eye development across metazoans (Pichaud and Desplan, 2002; Kozmik, 2005) and the transcription factor Tin/Nkx2.5 that is involved in heart development across bilaterians (Harvey, 1996; Holland et al., 2003; Davidson, 2006). Apparently, these genes were key regulators of ancestral GRNs that initiated the development of these organs. However, it is not clear why the developmental role of these particular genes is so well conserved and whether the transcriptional regulation of these genes is as conserved as their developmental roles.

In order to shed light on these fundamental questions we consider here a particular example, the forkhead transcription factor, Foxa. We review the conserved developmental role of Foxa and the transcriptional regulation of *foxa* orthologs in different organisms. Foxa is critical for similar morphogenetic processes in the digestive tracts of the mouse (Burtscher and Lickert, 2009), sea urchin (Oliveri et al., 2006) and nematode (Horner et al., 1998) embryos, and in the notochord formation in chordates (Friedman and Kaestner, 2006; Kumano et al., 2006; Kim et al., 2007; Passamaneck et al., 2009). A recent study reveals the *cis*-regulatory code that controls *foxa* expression in the sea urchin embryo (Ben-Tabou de Leon and Davidson, 2010). Detailed functional analyses of the regulation of *foxa* ortholog were done in the nematode, *Caenorhabditis elegans,* and some regulatory information is available for other species. We review this information and try to understand why *foxa* developmental role is so highly conserved and whether its transcriptional regulation as conserved as its developmental role.

Foxa conserved developmental role in regulating mesenchymal-to-epithelial transition

The expression of *foxa* orthologs in the endoderm lineage is highly conserved across metazoans (Mango et al., 1994; Horner et al., 1998; Koinuma et al., 2000; Fritzenwanker et al., 2004; Suri et al., 2004; Friedman and Kaestner, 2006; Oliveri et al., 2006; Kimura-Yoshida et al., 2007; Boyle and Seaver, 2008; Burtscher and Lickert, 2009). In

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this section, we review the role of Foxa in early development in the species where it was extensively studied, i.e., sea urchin, ascidians, mouse, and nematode (see phylogenetic tree, Fig. 1).

In the sea urchin embryo, gut formation involves two major processes: local shifts in position of cells that form the archenteron wall and polarized motility of the cells as they rearrange and form tight junctions (Hardin, 1989; Barnet et al., Submitted). In other words, the gut is formed by the local movement of cells that climb on top of each other (intercalation) and then elongate and generate tight junctions to form epithelial sheet. These processes clearly involve the regulation of structural genes and cell adhesion molecules. When *foxa* is downregulated by the injection of morpholino antisense oligonucleotides, these processes do not occur, and there is a failure of gut formation (Oliveri et al., 2006). In addition, the embryo produces excess numbers of pigment cells that are mesenchymal mesoderm derivatives (Oliveri et al., 2006). The suppression of mesodermal fate in the endoderm is mediated, at least in part, by Foxa repression of the gene that encodes the transcription factor GCM (Oliveri et al., 2006), a key regulator of mesodermal fate in the sea urchin embryo (Ransick and Davidson, 2006). Foxa has two other known targets, it activates the transcription of the gene that encodes the ligand, Hedgehog, and it represses its own gene expression (Oliveri et al., 2006). However, these regulatory interactions cannot explain the severe morphological phenotype of *foxa* downregulation. Most likely, *foxa* regulates structural genes and cell adhesion molecules that are necessary for intercalation and elongation. This is similar to its role in the definite endoderm (Burtscher and Lickert, 2009) and the notochord (Ang and Rossant, 1994) of the mouse and the pharynx of *C. elegans* (Gaudet and Mango, 2002; Mango, 2009), as explained below.

Mammals have three orthologs of *foxa* (Friedman and Kaestner, 2006). *foxa* ortholog, *foxa2*, plays a critical role in the specification and morphogenesis of the definitive endoderm (Burtscher and Lickert, 2009) and of the notochord (Ang and Rossant, 1994; Friedman and Kaestner, 2006). The *foxa2* gene is the first of *foxa* orthologs to be activated during mouse embryogenesis, and its expression is detected in the anterior primitive streak and the node (Friedman and Kaestner, 2006). *foxa2* is expressed in mesoderm and definitive endoderm cells migrating from the node, and the expression is maintained in the notochord and throughout the definitive endoderm (Sasaki and Hogan, 1993; Friedman and Kaestner, 2006; Burtscher and Lickert, 2009). A recent study has demonstrated explicitly the role of *foxa2* in the definitive endoderm morphogenesis, as follows (Burtscher and Lickert, 2009). At the onset of gastrulation, cells from the definitive endoderm migrate individually and intercalate into the visceral endoderm. Once the cells are within the visceral endoderm, they elongate and form tight cell junctions and epithelial sheet. In *foxa2* mutant mice these processes do not take place. Most cells do not intercalate, and if they do penetrate to the visceral ectoderm, they do not polarize nor elongate, and tight junctions are not formed (Burtscher and Lickert, 2009). In these cells, the adherent junction proteins E-cadherins and ZO-1 fail to aggregate in the cell junctions.

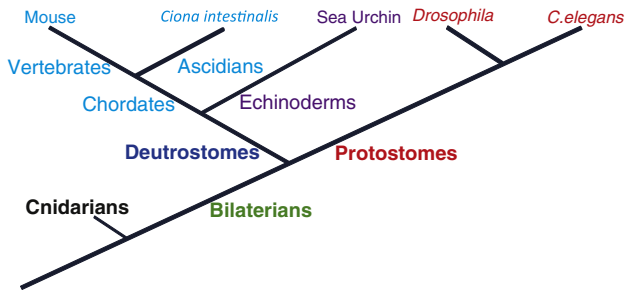

Fig. 1. Simplified phylogenetic tree of the organisms discussed in this review in the context of Foxa conserved developmental role and the transcriptional regulation of *foxa* orthologes.

The expression of *claudin4*, an important cell-adhesion molecule, vanishes in *foxa2* mutants (Burtscher and Lickert, 2009). Claudins bind specifically to ZO-1 so *foxa2* transcriptional regulation of *claudin4* might explain the failure of ZO-1 aggregation. Another direct target of *foxa2* in the definitive endoderm is the gene that encodes the transcription factor Gata4, a key endoderm regulator (Rojas et al., 2010). All in all, these studies reveal the important role of *foxa2* in controlling intercalation, elongation, and formation of epithelial tissue in the definitive endoderm of the mouse by the regulation of regulatory and structural genes that are critical for these processes.

Later in mouse development, *foxa* orthologs, *foxa1* and *foxa2*, are required for normal development of endoderm-derived organs such as the pancreas and the lungs (Wan et al., 2005; Friedman and Kaestner, 2006). Interestingly, recent studies show that *foxa2* functions as a suppressor of tumor metastasis by inhibition of epithelial-to-mesenchymal transition in human lung and pancreatic cancers (Song et al., 2010; Tang et al., 2010). In human lung cancer cells, Foxa2 directly represses the gene that encodes the transcription factor Slug (Tang et al., 2010), a key factor in epithelial-to-mesenchymal transition. This could be a part of the regulatory control that *foxa* mandates to promote epithelial formation and prevent mesenchymal fate. Apparently, the regulatory role that *foxa* plays through development is relevant to the normal function of differentiated adult cells that express *foxa* orthologs.

foxa2 plays a role in the notochord formation across chordates (Ang and Rossant, 1994; Weinstein et al., 1994; Ruiz i Altaba et al., 1995; Shimeld, 1997; Friedman and Kaestner, 2006; Yamanaka et al., 2007). The notochord is a mesoderm derivative that is a hallmark of all chordates (Stemple, 2004). In its final form, the notochord is a rod of large cells positioned between the developing spinal cord and gut (Stemple, 2004). The notochord produces a variety of secreted signaling factors, such as Sonic hedgehog, which induce particular specification states in the notochord surrounding tissues (Stemple, 2004). Similarly to the definitive endoderm in the mouse, the notochord is formed through cells intercalation, elongation, and formation of tight junctions (Munro and Odell, 2002; Yamanaka et al., 2007). In *foxa2* mutant mouse, the notochord is completely missing (Ang and Rossant, 1994; Yamanaka et al., 2007), indicating a critical role of *foxa2* in regulating the formation of this organ. In the mouse embryo, Foxa2 drives the expression of the gene that encodes the T-box transcription factor, Brachyury, and together, Foxa2 and Brachyury drive *not* and *sonic hedgehog*, key notochord regulatory genes (Jeong and Epstein, 2003; Abdelkhalek et al., 2004; Yamanaka et al., 2007). Multiple studies show that Foxa and Brachyury directly regulate gene expression in the ascidian notochord, which suggests a conserved regulatory role of these two factors in the notochord development (Kumano et al., 2006; Passamaneck et al., 2009). This is another example of a developmental process that involves intercalation, elongation, and formation of epithelial tissue that is entirely abolished by the knock-out of *foxa* ortholog.

pha-4 is the ortholog of *foxa* in the nematode, *C. elegans*. *pha-4* is expressed in all the cells that generate the pharynx and the gut, that is, in all the daughters of the E cell (endoderm), and all the daughters of MS and AB cells that contribute to the pharynx (mesoderm) (Azzaria et al., 1996; Murray et al., 2008). During pharynx development, cells that originate from different lineages but are fated to form the pharynx, cluster together, ingress, and go through mesenchymal-to-epithelial transition (Mango, 2009). In *pha-4* mutants, the entire pharynx is deleted while other organs, including the gut, appear to be normal (Mango et al., 1994; Horner et al., 1998; Mango, 2009). The pharyngeal precursors that normally cluster together and ingress during gastrulation remain dispersed in the embryo surface when *pha-4* is mutated (Horner et al., 1998). This elimination of clustering and epithelialization is similar to *foxa2* phenotypes in other organisms discussed above. Different studies show that Pha-4 regulates many of the regulatory and structural genes that are necessary for the specification and

morphogenesis of the pharynx (Gaudet and Mango, 2002; Anokye-Danso et al., 2008; Mango, 2009), as well as represses ectoderm regulatory genes (Kiefer et al., 2007). Overall, Pha-4 is the central regulator of the pharynx development and its expression is necessary for this organ formation, in particular, for cells clustering, migration, and mesenchymal-to-epithelial transition.

It is important to note that the regulatory role of Foxa is not limited to intercalation and epithelialization nor can it be the only regulator of these processes. For example, Pha-4 has different regulatory roles later in *C. elegans* development and aging (Panowski et al., 2007; Chen and Riddle, 2008). On the other hand, the gut formation in *C. elegans* is unaffected in *pha-4* mutant so other factors are regulating epithelialization there. *foxa2* and other *foxa* orthologs in the mouse are important to various regulatory processes (see e.g., Kimura-Yoshida et al., 2007; Gao et al., 2008; Lin et al., 2009). Yet, the similarity of the phenotypes observed when *foxa* orthologs are knocked down in diverse organisms such as the mouse, the nematode, and the sea urchin is quite striking. This conserved role of *foxa* in embryogenesis raises the following question: is *foxa* regulation as conserved as its role in early development, or did it change to accommodate evolutionary changes that involve *foxa* function? The next sections describe *foxa* regulation in different organisms and shed light on this question.

foxa transcriptional regulation in the sea urchin embryo

A detailed *cis*-regulatory analysis was recently conducted to decipher the genomic code that controls *foxa* expression early in sea urchin development (Ben-Tabou de Leon and Davidson, 2010). Here we review the main results of this work. In the sea urchin embryo *foxa* is expressed in the presumptive mesoderm and endoderm. At mid-blastula stage, (about 15 h post fertilization (hpf)) *foxa* is expressed in both non-skeletogenic mesoderm (NSM) and endoderm precursors (Fig. 2A, B) (Peter and Davidson, 2010). At mesenchyme blastula stage (18–20 hpf), *foxa* expression is shut down in the NSM progenitors and transcription continues from then on only in the endoderm (Fig. 2A, B) (Peter and Davidson, 2010). Later, *foxa* is also expressed in a patch of cells in the oral ectoderm where the mouth will form (Fig. 2B). One of the important aspects of *foxa* regulation is the transition between the broad expression in both the NSM and endoderm to the specific expression in the endoderm. Understanding this transition can illuminate the mechanisms that control the endoderm versus mesoderm cell fate decision.

cis-regulatory analysis of *foxa* reveals that the spatial expression of *foxa* is controlled by Tcf-Groucho/β-catenin toggle switch (Fig. 2C, D) (Weitzel et al., 2004; Wikramanayake et al., 2004; Range et al., 2005; Ben-Tabou de Leon and Davidson, 2010). Tcf is a transcription factor that can act either as a repressor or as an activator, depending on its cofactors (Range et al., 2005; Ben-Tabou de-Leon and Davidson, 2007). When Tcf binds to β-catenin, they form a complex permissive for transcription, otherwise Tcf and the co-repressor Groucho form dominant repressor complex. Therefore, Tcf targets are expressed only in cells where β-catenin is nuclear localized and repressed elsewhere. Maternal anisotropies in the sea urchin egg lead to β-catenin nuclearization in the vegetal cells of the embryo early in development (Logan et al., 1999; Weitzel et al., 2004). By mid-blastula stage nuclear β-catenin has cleared from the skeletogenic mesoderm (SM) nuclei and is localized in the veg2 lineage nuclei, i.e., in cells that give rise to NSM plus endoderm (Fig. 2C, Logan et al., 1999). At this time, *foxa* expression is likewise restricted to these cells (Fig. 2B,C, Peter and Davidson, 2010). At mesenchyme blastula stage β-catenin clears from the NSM nuclei as well and remains visible only in the nuclei of veg2 and veg1 endoderm (Fig. 2C) (Logan et al., 1999). This leads to silencing of *foxa* expression in the NSM due to Tcf-Groucho repression there. Expression of *foxa* is henceforth restricted to the veg2 endoderm (Fig. 2B, C). Thus the Tcf-Groucho/β-catenin system initially enables broad *foxa* expression throughout the veg2 endomesoderm and later restricts it to the endodermal domain of this lineage.

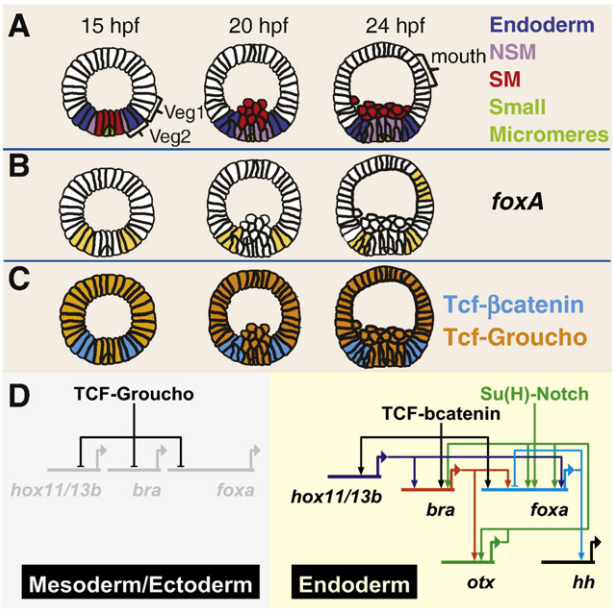

Fig. 2. *foxa* expression and *cis*-regulation in the sea urchin embryo (Ben-Tabou de Leon and Davidson, 2010). A. Lineage fate map showing lateral view of the sea urchin embryo at 15, 20, and 24 hpf. The SM lineage is marked in red, the NSM lineage is marked in purple, veg2 endoderm lineage is marked in blue, and veg1 and the ectoderm lineages are marked in white. The most vegetal descendents of veg1 contribute to the endoderm. B. *foxa* spatial expression at 15, 20, and 24 hpf. C. Diagrams of Tcf activity mode at 15, 20, and 24 hpf. The cells where Tcf binds to the co-repressor Groucho to form a repressive complex are marked in orange. The cells where Tcf binds to β-catenin to form a permissive complex are marked in cyan. D. Diagram of the gene regulatory network that drives *foxa* expression in the sea urchin embryo. Tcf-Groucho represses *foxa* and other endodermal genes in the ectoderm and progressively in the mesoderm. The multiple additive activators that contribute to *foxa* expression are Hox11/13b, Su(H)-Notch, Otx, and Brachyury. Foxa represses its own gene expression.

foxa is activated by multiple additive inputs (Fig. 2D) (Ben-Tabou de Leon and Davidson, 2010). One of the early activators of *foxa* is the transcription factor Hox11/13b that is co-expressed with *foxa* at blastula stage (Peter and Davidson, 2010). Later in development *hox11/13b* expression turns off in veg2, and the gene becomes active in veg1, where *foxa* is not expressed. At blastula stage *foxa* is also a target of the Delta–Notch signaling that occurs in veg2 and its NSM descendents due to reception of the Delta ligand produced by the adjacent SM cells (Fig. 2D) (Ben-Tabou de Leon and Davidson, 2010). At mesenchyme blastula stage, the transcription factors Otx and Brachyury boost *foxa* expression and Foxa represses its own gene expression (Fig. 2D) (Ben-Tabou de Leon and Davidson, 2010). Different Otx isoforms are expressed everywhere in the embryo throughout development. Therefore, the Otx input probably acts to boost *foxa* level, providing no spatial information. *brachyury* is co-expressed with *foxa* starting at blastula stage (Peter and Davidson, 2010). At mesenchyme blastula stage *brachyury* expression begins to fade in veg2 and becomes active in the veg1 ring of cells. Thus after 24 hpf the expression domains of these genes have a small overlap.

Summing up *foxa cis*-regulation at early development of the sea urchin embryo, *foxa* is regulated by the activators Hox11/13b, Su(H), Brachyury, and Otx, which act additively and partially overlap in time and embryonic space with *foxa* expression. Foxa is an auto-repressor. *foxa* expression is restricted spatially by Tcf-Groucho repression that prevents expression in the ectoderm and progressively in the mesoderm. In regulatory logic terms, *foxa cis*-regulatory modules execute additive OR logic on all its positive inputs, and NOT logic on Tcf-Groucho.

The transcriptional regulation of *foxa* ortholog, *pha-4*, in *C. elegans*

As described above, *pha-4*, the *C. elegans* ortholog of *foxa*, is expressed in the gut and in the pharynx of the nematode (Horner et al., 1998). The direct regulation of *pha-4* in *C. elegans* was not studied by a *cis*-regulatory analysis. Yet, a comprehensive study of mutants and knock-down phenotypes enabled the construction of a model of the endomesoderm GRN, which includes *pha-4* predicted connections (Owraghi et al., 2010). Based on this work, we present a partial GRN diagram that highlights the predications for *pha-4* inputs in different cell lineages, Fig. 3 (Owraghi et al., 2010).The gut is formed from all the daughters of the E cell. Reception of Wnt/MapK signaling in the E cell activates the orthologs of the Tcf–β-catenin switch (Pop1-Sys1), and as a result, the genes encoding the GATA factors End-1 and End-3 are turned on in this cell (Owraghi et al., 2010). These genes are repressed by Tcf-Groucho in the MS cell that does not receive the Wnt/MapK signaling. Apparently, *pha-4* does not have a direct link to Tcf since it is expressed in both the E and the MS cells. In the descendents of the E cell, End-1 and End-3 activate the genes encoding the GATA factors Elt-2 and Elt-7, which then form a positive feedback regulatory loop and activate the expression of the *pha-4* gene (Fig. 3).

Regulatory roles of Tcf–β-catenin and GATA factors in the endoderm specification were observed in other organisms. Gata4/5/6 family of transcription factors plays a central role in the endoderm specification in vertebrates (Soudais et al., 1995; Bossard and Zaret, 1998; Capo-Chichi et al., 2005; Zorn and Wells, 2007) and *Drosophila* (Murakami et al., 2005). As stated above, in the sea urchin embryo, the Tcf–β-catenin switch restricts the spatial expression of *foxa* and other endodermal genes (Fig. 2D) (Ben-Tabou de Leon and Davidson, 2010). In the embryo of the chordate, *Ciona intestinalis*, β-catenin is important to the endoderm specification (Imai et al., 2000). Deletion of β-catenin in the mouse definitive endoderm changes cell fate from endoderm to precardiac mesoderm and leads to the formation of multiple hearts (Lickert et al., 2002). The widely shared role of GATA factors and Tcf-Groucho/β-catenin in the endoderm specification suggests that these factors are part of an ancestral endoderm GRN. The GRN that controls the E cell lineage seems to be a derived form of this ancestral GRN. However, the GRN that controls the pharynx formation is significantly different.

In the daughters of the MS cell that contributes to the pharynx, *pha-4* is downstream of the T-box transcription factor Tbx35 and the Nk-2 class homeodomain transcription factor, Ceh-51 (Fig. 3) (Owraghi et al., 2010). These two factors are expressed in the MS lineage and are essential to the production of MS-derived tissues (Broitman-Maduro et al., 2009). The MS cells secrete the ligand Delta and its reception activates *pha*-4 expression in the AB-cell daughters that contribute to the pharynx (Fig. 3) (Owraghi et al., 2010). In these cells, the T-box transcription factors, Tbx37 and Tbx38, contribute to the expression of *pha-4* (Fig. 3). In AB daughter cells, Pha-4 activates the gene that encodes the T-box transcription factor, Tbx-2 (Fig. 3) (Smith and Mango, 2007). Tbx-2 feeds back and activates *pha-4* expression so the two genes form a positive feedback loop essential for the maintenance of *pha-4* expression (Smith and Mango, 2007). Interestingly, the regulatory interaction between *foxa* orthologs to T-box transcription factors is common to the AB and MS cells in *C. elegans*, the chordate notochord, and the endoderm of the sea urchin. The T-box transcription factor, Brachyury, interacts with Foxa orthologs in the chordate notochord and in the sea urchin endoderm (Jeong and Epstein, 2003; Abdelkhalek et al., 2004; Hotta et al., 2008; Tamplin et al., 2008; Ben-Tabou de Leon and Davidson, 2010). The reason for this similarity could be the ancestral regulatory role of T-box transcription factors, particularly Brachyury (Technau, 2001; Marcellini et al., 2003; Hotta et al., 2008) in controlling morphogenetic processes in cooperation with Foxa.

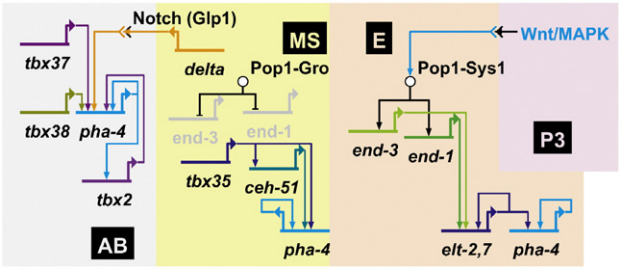

Fig. 3. The predicted transcriptional regulation of *foxa* ortholog, *pha-4*, in the nematode *C. elegans* (based on Owraghi et al., 2010). Different lineages are indicated by different background colors. Pha-4 is expressed in all the daughters of the E cell and in the daughters of the AB and MS cells that give rise to the pharynx. Pop1 is ortholog of Tcf; Sys1 is ortholog of β-catenin; End-1, End-3, Elt-2, and Elt-7 are GATA transcription factors. In each lineage, *pha-4* is activated by different set of transcription factors. In all the lineages, Pha-4 positively regulates its own gene expression.

Pha-4 positively regulates its own gene expression in all the cells where it is expressed, but it is not clear whether this regulatory interaction is direct or indirect. The regulatory control of *pha-4* in the MS and AB cells seems to be *C. elegans*-specific, except from the Delta–Notch input. As stated above, the reception of the SM Delta signal contributes to *foxa* expression in the NSM lineage in the sea urchin (Fig. 2D) (Ben-Tabou de Leon and Davidson, 2010). It is hard to conclude about the evolution of Delta–*foxa* regulatory connection based on echinoderm and nematodes only. Without further evidence for Delta activation of *foxa* orthologs in other organisms, this link seems to be the result of convergent evolution and not a part of an ancestral GRN.

All in all, *pha-4* is activated by different activators in each of the three lineages that form the gut and the pharynx in *C. elegans*. It appears that *pha-4* does not have a direct link to Tcf so its expression outside of the E cell lineage is not restricted by Tcf-Groucho repression. Apparently, *pha-4* gained new activators that drive its expression in the cells that form the pharynx. The absence of a restricting repressor and the gain of new activators allow the expression of *pha-4* in mesodermal lineages where *pha-4* drives its distinctive developmental program.

Discussion

The embryo morphologies of the sea urchin, the mouse, and nematode, *C. elegans*, are significantly different. Yet, in all these organisms, orthologs of *foxa* are expressed in cells that intercalate, polarize, and form tight junctions. The loss of *foxa* expression eliminates these morphogenetic processes. The transcriptional regulation of *foxa* in *C. elegans* is quite different from its regulation in the sea urchin embryo (compare Fig. 3 to Fig. 2D). *foxa* activating inputs are not only different between the two organisms, but in the case of *C. elegans*, unique in every lineage where *pha-4* is expressed (Fig. 3). Apparently, the upstream regulation of *foxa* is rather flexible while the downstream phenotypes are highly conserved. Davidson and Erwin recently suggested that basal ancestral GRNs were shallow and consisted of few regulatory genes that controlled batteries of structural genes (Davidson and Erwin, 2009). They propose that evolution had added regulatory links into the GRN of each species, to either advance the network function or to redeploy the ancestral GRN into a new embryonic address. Taking this view and the evidence presented above, Foxa might have been a member of such ancestral GRN that controlled structural genes necessary for cell motility and migration and for mesenchymal-to-epithelial transition. *foxa* regulation had changed through evolution according to the developmental program in each species so it plays its role controlling morphogenesis at the relevant embryonic lineage.

An apparent example of regulatory changes that enabled the activation of *foxa* in a new embryonic territory is the activation of *pha-4* in the pharynx of the *C. elegans*. *pha-4* is driven by a specific set of

transcription factors in the MS lineage and a different set of transcription factors specific to the AB lineage. This regulatory code enabled the mesodermal expression of *pha-4* and the cooption of the developmental program that it drives. Evolutionary modification of the regulatory code must have led to *foxa2* expression in the notochord, a chordate mesoderm derivative (Ang and Rossant, 1994; Weinstein et al., 1994; Yamanaka et al., 2007).

The logic applied on *foxa* inputs might have contributed to the flexibility of *foxa* regulation. In the sea urchin embryo *foxa* expression is regulated by a combination of multiple additive inputs, that is, OR logic (Istrail and Davidson, 2005; Istrail et al., 2007; Ben-Tabou de Leon and Davidson, 2010). In *cis*-regulatory modules that execute OR logic, loss of a binding site does not eliminate the expression of the downstream gene since other inputs are still driving it. Furthermore, in Or logic, an addition of a functional binding site does not require the presence of other specific binding sites. On the other hand, in *cis*-regulatory modules that execute AND logic (multiple necessary inputs (Istrail and Davidson, 2005; Istrail et al., 2007)), loss of any binding site causes complete loss of the downstream gene expression. Gain of function in AND logic can only happen when there is a gain of binding sites of all the necessary inputs. Therefore, in principle, OR logic might be more flexible to evolutionary changes of addition or deletion of binding sites. If the logic applied on *foxa* inputs in other organisms is also OR logic, it might have facilitated the evolutionary flexibility of *foxa* transcriptional regulation. It would be illuminating to identify the logic applied on *foxa* inputs in other organisms and learn about the role of regulatory logic in GRN evolution.

The connection between the evolution of developmental GRN to the evolution of diverse body plans is more and more evident as models of GRNs of diverse organisms become available (Davidson and Erwin, 2009; Erwin and Davidson, 2009). It is now apparent that different parts of GRNs evolve at a different pace and some parts are more conserved than others (Hinman et al., 2003; Hinman and Davidson, 2007; Davidson and Erwin, 2009; Erwin and Davidson, 2009; McCauley et al., 2010). Here we focused on one regulatory gene and tried to understand the reasons for its highly conserved developmental role and whether its regulation is as conserved as its role. We learned that Foxa orthologs in a wide spectrum of organisms are essential to a specific morphogenetic process: cells intercalation followed by elongation and epithelialization. Foxa controls, this process not only in the endoderm but also in the mesoderm of some organisms. The extreme conservation of Foxa developmental role is most likely due to its ancestral direct control of batteries of structural genes that mandate mesenchymal-to-epithelial transition and initially enabled the gut formation. Foxa regulation had changed through evolution to accommodate *foxa* expression at novel embryonic addresses, for example, the *C. elegans* pharynx and the chordate notochord, both mesodermal derivatives. That is, evolution had added layers of regulatory control in order to either redeploy or improve the function of the conserved morphogenetic program controlled by Foxa.

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